

- ing the amino acids SEKDEL (Fig. 1A) were annealed and ligated at the Spe I site of the gene encoding LT-B in pJC217 to obtain pLTK217. The LT-B coding regions from pJC217 and pLTK217 were subcloned into pBluescript KS (Stratagene) at Eco RI-Hind III, then excised with Bam HI-Dra I and ligated into pBT210 to form pLTB200 and pLTK200, respectively. The Sac I fragments of pLTB200 and pLTK200 were ligated into pLTB5-Sac I to give pLTB210 and pLTK210. The Hind III-Eco RI cassettes in pLTB210 and pLTK210 were transferred to the *Agrobacterium* T-DNA vector pBI101 [R. A. Jefferson *et al.*, *EMBO J.* 13, 3901 (1987)] to give pLTB110 and pLTK110.
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Generation and Assembly of Secretory Antibodies in Plants

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Four transgenic *Nicotiana tabacum* plants were generated that expressed a murine monoclonal antibody kappa chain, a hybrid immunoglobulin A-G heavy chain, a murine joining chain, and a rabbit secretory component, respectively. Successive sexual crosses between these plants and filial recombinants resulted in plants that expressed all four protein chains simultaneously. These chains were assembled into a functional, high molecular weight secretory immunoglobulin that recognized the native streptococcal antigen I/II cell surface adhesion molecule. In plants, single cells are able to assemble secretory antibodies, whereas two different cell types are required in mammals. Transgenic plants may be suitable for large-scale production of recombinant secretory immunoglobulin A for passive mucosal immunotherapy. Plant cells also possess the requisite mechanisms for assembly and expression of other complex recombinant protein molecules.

Secretory immunoglobulin A (SIgA) is the most abundant form of immunoglobulin (Ig) in mucosal secretions, where it forms part of the first line of defense against infectious agents. The molecule exists mainly in the 11S dimeric form, in which two monomeric IgA antibody units are associated with the small polypeptide joining (J) chain and with a fourth polypeptide, secretory component (SC). The ability to pro-

duce monoclonal SIgA would be of substantial value, but the synthesis is complicated because it requires plasma cells secreting dimeric IgA (dIgA) as well as epithelial cells expressing the polymeric Ig receptor (pIgR). Normally, pIgR on the epithelial basolateral surface binds dIgA, initiating a process of endocytosis, transcytosis, phosphorylation, proteolysis, and ultimate release of the SIgA complex at the apical surface into the secretion (1). Here, we focused on the ability of transgenic plants to assemble secretory antibodies.

Genes encoding the heavy and light chains of a murine antibody (Guy's 13), a murine J chain, and a rabbit SC were introduced into separate transgenic tobacco plants. Guy's 13 is a murine IgG1 monoclonal antibody (mAb) that recognizes the streptococcal antigen (SA) I/II cell surface adhesion molecule of *Streptococcus mutans*

and *S. sobrinus* (2). Transgenic full-length Guy's 13 has been generated in *N. tabacum* plants and was found to be correctly assembled (3). Modification of the heavy chain by replacement of its C γ 3 domain with C α 2 and C α 3 domains from an IgA-secreting hybridoma (MOPC 315) did not affect the assembly or function of the antibody (IgA-G) produced in transgenic plants (3). Protein immunoblot analysis (4) of the IgA-G plant extract with antiserum to the κ light chain under nonreducing conditions showed a band of ~210 kD, which is consistent with the presence of the extra constant region domains in the IgA-G antibody construct as compared with the original IgG1 antibody (Fig. 1A, lanes 1 and 3). A number of smaller proteolytic fragments were also detected, which is consistent with previous findings (3). A mouse J chain construct that consisted of coding-length complementary DNA (cDNA) was amplified with synthetic oligonucleotide primers corresponding to the NH $_2$ -terminal MKTHLL and the COOH-terminal SCYPD sequences of the mouse J chain (5). The SC construct used in this study consisted of coding-length cDNA amplified with synthetic oligonucleotide primers corresponding to the NH $_2$ -terminal MALFLL sequence and the AVQSAE sequence near the COOH-terminus of rabbit pIgR (6). Transgenic plants were then regenerated (7).

The plants that expressed the J chain were crossed with those expressing IgA-G. The progeny showed a second major Ig band at ~400 kD, approximately twice the relative molecular mass of the IgA-G molecule (Fig. 1A, lane 4), which suggested that a dimeric antibody (dIgA-G) had been assembled. Mature plants that expressed dIgA-G were crossed with a homozygous plant that expressed SC. The progeny plants (SIgA-G) included those that produced a higher mo-

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lecular mass band of ~470 kD in protein immunoblot analysis under nonreducing conditions (Fig. 1A, lane 5); such a molecular size is consistent with that expected for a secretory Ig. Detection with antiserum to SC confirmed that this high molecular mass protein contained SC (Fig. 1A, lane 7). The plant extracts also contained the 400-kD

band (dIgA-G) and the 210-kD band (IgA-G), but these were detected only by antiserum to the κ light chain and not by antiserum to SC. In the transgenic plant that secreted SC alone, no high molecular mass proteins were detected in protein immunoblotting under nonreducing conditions (Fig. 1A, lane 9), and hence there was no evidence that SC assembled with endogenous plant proteins or formed multimers.

Further protein immunoblot analysis under reducing conditions demonstrated that extracts from the plants that expressed antibodies (IgA-G, dIgA-G, and SlgA-G), but not those that expressed the J chain or SC, contained identical antibody heavy and light chains (Fig. 1B, upper and middle panels). Only the SC and SlgA-G plants expressed proteins that were recognized by antiserum to SC (lower panel). The dissociation of SC from Ig heavy chains only under reducing conditions suggests that the SC chain was at least partially covalently linked in the assembled SlgA-G molecule. The molecular mass of the major SC band under reducing conditions is ~50 kD, which is lower than expected (66.5 kD). This is probably a result of proteolysis, which may occur in the intact plant or during sample preparation. SC bound to dimeric IgA is often found proteolyzed to smaller but biologically active forms in vivo (8). However, in the protein immunoblot analysis under nonreducing conditions, the molecular mass difference between dIgA-G and SlgA-G was ~70 kD, as expected (Fig. 1A). No cross-reacting proteins were detected in extracts from the wild-type control plant.

In mammals, the assembly of SC with antibody requires the presence of the J chain (9); we next investigated whether this was also the case in plants. Plants expressing

monomeric IgA-G were crossed with SC-expressing plants. In the progeny, only the 210-kD monomeric form of the antibody was recognized by antiserum to the κ light chain (Fig. 2A, lanes 1 and 2); antiserum to SC recognized free SC but did not recognize proteins associated with Ig (Fig. 2B). This result was confirmed in all 10 plants examined, whereas all 10 plants that coexpressed the J chain, the antibody chains, and SC assembled the 470-kD SlgA-G molecule. This finding confirms the requirement of the J chain for SC association with Ig and suggests that the nature of the association in plants is similar to that in mammals.

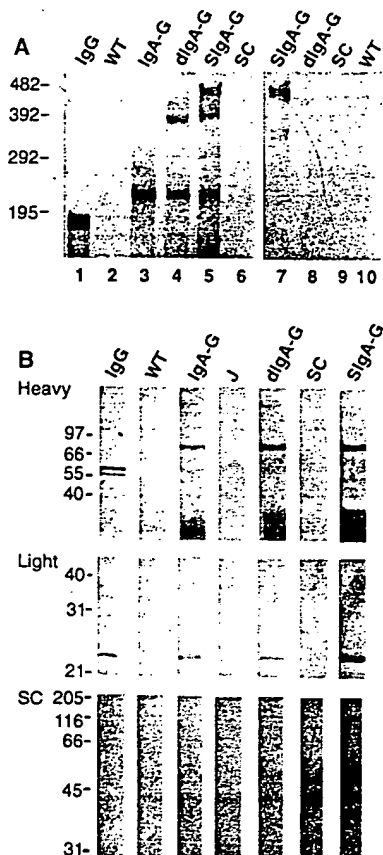


Fig. 1. (A) Protein immunoblot of plant extracts prepared under nonreducing conditions, detected with antisera to the mouse κ light chain (lanes 1 to 6) or to rabbit SC (24) (lanes 7 to 10). Samples were prepared (4) and separated on 4% SDS-polyacrylamide electrophoresis gel (SDS-PAGE). (B) Protein immunoblot of plant extracts prepared under reducing conditions. Samples were prepared as in (4), but with the addition of 5% β -mercaptoethanol. SDS-PAGE in 10% acrylamide was performed and the gels were blotted as before. Detection was with antisera to the mouse γ 1 heavy chain (upper panel), the mouse κ light chain (middle panel), or rabbit SC (lower panel), followed by the appropriate second-layer alkaline phosphatase-conjugated antibody. IgG, Guy's 13 mAb (2) prepared in hybridoma cell culture supernatant; WT, nontransformed wild-type plant; IgA-G, transgenic plant expressing modified heavy and light chain genes of Guy's 13; dIgA-G, transgenic plant expressing modified heavy and light chain genes of Guy's 13 and the J chain; SlgA-G, transgenic plant expressing modified heavy and light chain genes of Guy's 13, the J chain, and SC; SC, transgenic plant expressing SC; and J, transgenic plant expressing the J chain. Molecular masses are indicated in kilodaltons.

Fig. 2. Coexpression of IgA-G with SC. Protein immunoblotting of transgenic plant extract (4) was performed (A) under nonreducing conditions on 4% SDS-PAGE and detected with goat antiserum to the κ light chain, followed by alkaline phosphatase-labeled rabbit antiserum to goat IgG, and (B) under reducing conditions on 10% SDS-PAGE and detected with sheep antiserum to SC, followed by alkaline phosphatase-labeled donkey antiserum to sheep IgG. IgA-G-SC, transgenic plant expressing modified heavy and light chain genes of Guy's 13 and SC.

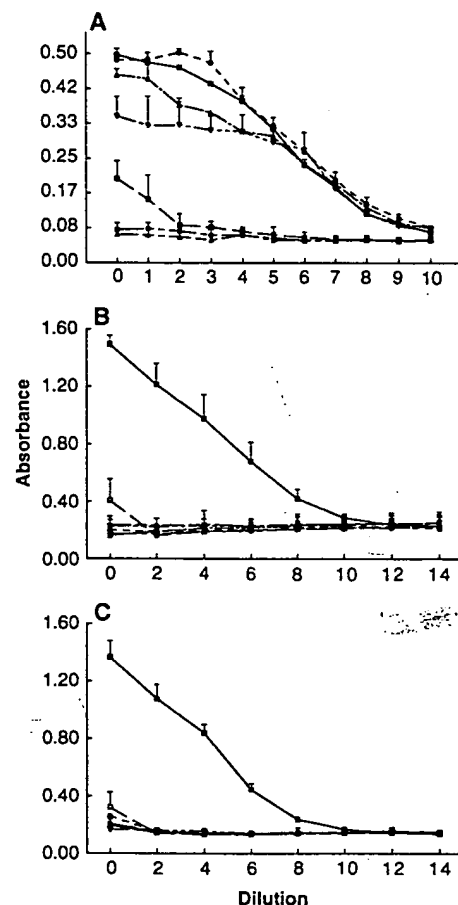
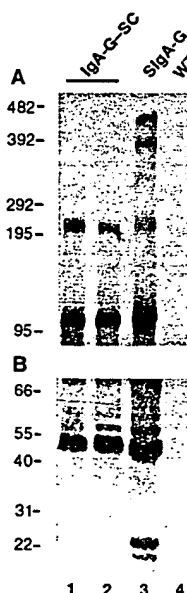


Fig. 3. Functional antibody expression in transgenic *N. tabacum*, as measured by absorbance at 405 nm (A_{405}). (A) Plant extract binding to purified SA I/II, detected with HRP-labeled antiserum to the κ light chain. (B) Plant extract binding to purified SA I/II, detected with sheep antiserum to SC followed by alkaline phosphatase-labeled donkey antiserum to sheep Ig. (C) Plant extract binding to streptococcal cells, detected with sheep antiserum to SC followed by alkaline phosphatase-labeled donkey antiserum to sheep Ig. Guy's 13 hybridoma cell culture supernatant (IgG) was used as a positive control. The initial concentration of each antibody solution was 5 μ g/ml. Dilution numbers represent serial double dilutions. Results are mean \pm SD of three separate triplicate experiments. ■, SlgA-G; ●, dIgA-G; ▲, IgA-G; □, SC; ○, J; △, WT; and ▼, Guy's 13.

Functional antibody studies were carried out with the five plant constructs by enzyme-linked immunosorbent assay (ELISA) (Fig. 3) (10). All plants expressing antibody light and heavy chains assembled functional antibodies that specifically recognized SA I/II (Fig. 3A). The levels of binding and titration curves were similar to those of the native mouse hybridoma cell supernatant. No SA I/II binding was detected with wild-type plants or with plants expressing the J chain or SC. The binding of antibody to immobi-

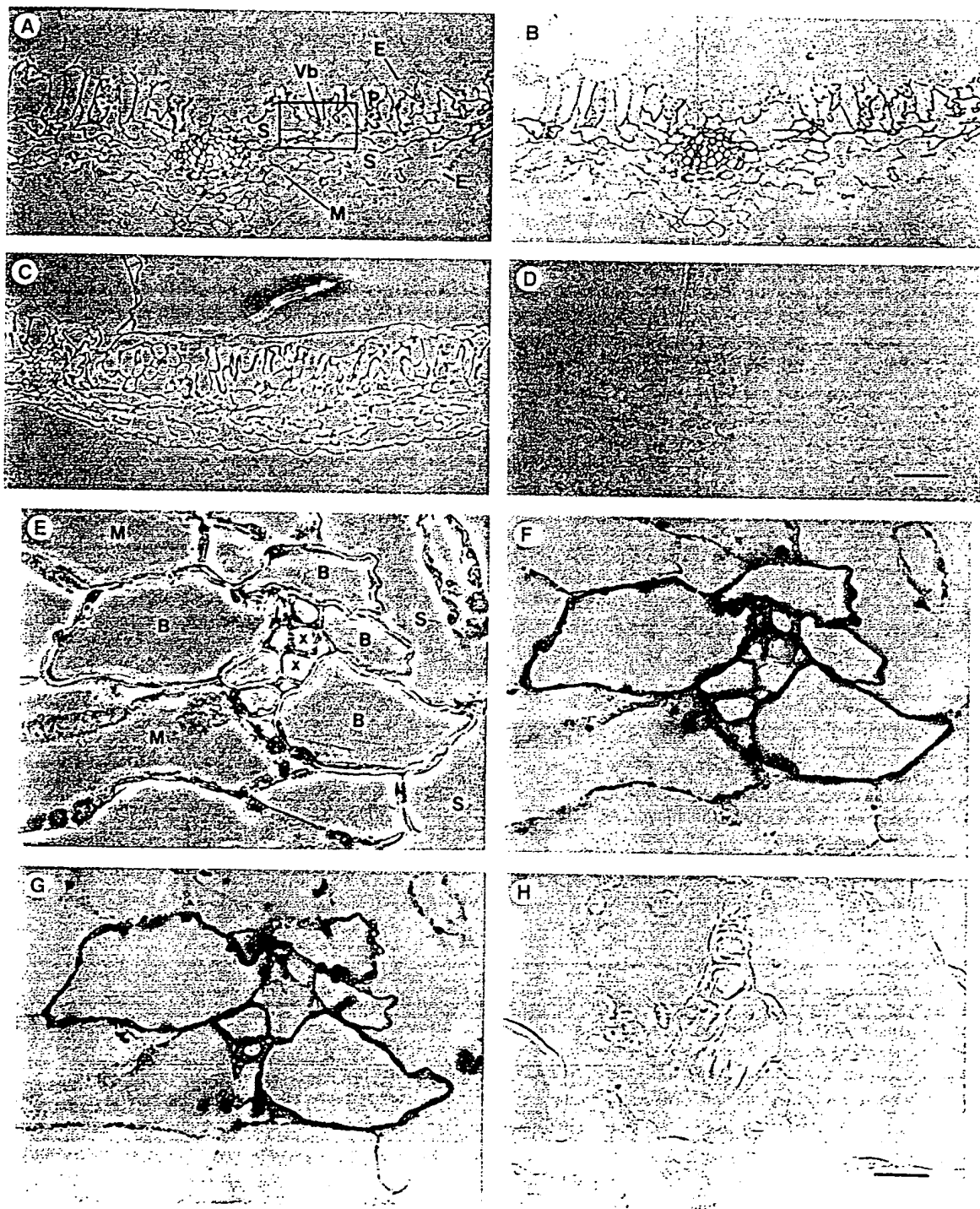
lized purified SA or native antigen on the bacterial cell surface was also detected with antiserum to SC (Fig. 3, B and C). In these assays, only the SIgA-G plant antibody binding was detected and not the functional antibodies in the IgA-G or dIgA-G plants. These results confirm that SC was assembled with antibody in the SIgA-G plant but did not interfere with antigen recognition or binding.

The assembly of functional Ig molecules in plants is very efficient (11). Initial esti-

mates for the plants expressing SIgA-G suggest that approximately 50% of the SC is associated with dimeric IgA-G in the plant extracts (12). Preliminary results indicate that the SIgA-G yield from fully expanded leaf lamina is 200 to 500 μ g per gram of fresh weight material. This yield is considerably greater than that determined for monomeric IgA-G and is consistent with the suggestion that SIgA-G might be more resistant to proteolysis.

Here, the fidelity of plant assembly has

Fig. 4. Photomicrographs of transgenic SIgA-G and control *N. tabacum* leaf tissue with immunogold detection of murine α chain or rabbit SC (16). (A) Phase contrast view of SIgA-G leaf in cross section. (B) Bright field view of the same leaf cross section as (A), showing immunogold localization of antibody heavy chain. Immunolocalization of SC on a section serial to that presented in (A) and (B) showed the same cellular localization as for antibody heavy chain (12). (C) Phase contrast view of a control leaf cross section from a transgenic plant that did not contain any Ig coding sequences. (D) Bright field view of the same leaf cross section as (C), showing the absence of immunogold localization of antibody heavy chain. (E) Phase contrast illumination of a minor vascular bundle [framed area of (A)]. (F) Bright field illumination of the same field as (E), showing immunogold localization (black) of SC. (G) Bright field illumination of a serial leaf cross section of the vascular bundle, demonstrating the same immunogold localization (black) of antibody heavy chain as that shown in (F). (H) Bright field illumination of a control leaf vascular bundle in cross section, showing absence of immunogold localization of antibody heavy chain. A control leaf section was also incubated with the gold-labeled secondary antibodies alone, and no binding was observed (12). M, spongy mesophyll cells; E, epidermal cells; S, intercellular spaces; P, cascade parenchyma cells; Vb, vascular bundles; X, xylem tracheids; and B, bundle sheath cells. Scale bars: 100 μ m (A through D), 10 μ m (E through H).



been extended to include dimerization of monomeric antibody by the J chain. Coexpression of recombinant IgA with the J chain through the use of baculovirus in insect cells has been reported (13); however, only a small proportion of the expressed antibody was dimerized, and most remained in a monomeric form. By contrast, in plants the dimeric antibody population represents a major proportion (~57%) of the total antibody (Fig. 1A, lane 4). This is also the first report of an assembled secretory antibody (SlgA-G) that binds as well to the corresponding antigen as does the parent mAb and constitutes a major proportion of the total assembled antibody (~45%; Fig. 1A, lane 5). Protein immunoblot analysis potentially underestimates the total extent of assembly of SlgA-G because it only detects antibody that is covalently linked to SC, whereas SlgA can occur in vivo as a mixture of covalently and noncovalently linked molecules (14).

The four transgenes for SlgA-G were introduced into plants with the identical pMON530 expression cassette, native leader sequences, and a promoter sequence derived from the 35S transcript of the cauliflower mosaic virus, which directs expression of transgenes in a variety of cell types of most plant organs (15). The use of the same promoter for all four transgenes maximized the likelihood of coincidental expression in a common plant cell. Microscopic observation of SlgA-G plants (16) revealed that many cell types of the leaves contained SlgA-G components (Fig. 4, A and B). The predominant accumulation of these proteins was in the highly vacuolated cells of the mesophyll, particularly in bundle sheath cells; the cytoplasmic band surrounding the large central vacuole was strongly labeled (Fig. 4, F and G). At the level of light microscopy, it is not possible to distinguish between antigens that are cytoplasmic and those that are contained in the luminal apoplastic space between the cell wall and the plasmalemma, but it is evident that the recombinant antibody components do not penetrate the cell wall.

Restriction of the largest SlgA-G components, SC and heavy chain, within the confines of the protoplasmic or apoplastic compartments of individual cells would constrain the assembly of Slg to single cells. In contrast, two cell types are required to produce SlgA in mammals. In the plant system, a mature SC devoid of signals for membrane integration, transcytosis, or subsequent proteolysis can thus be assembled with a hybrid Ig containing α domains within the secretory pathway of the cell. Assembly of monomeric antibody is known to require the targeting of both light and heavy chains to the endoplasmic reticulum (ER) (17). Thus SlgA-G assembly might occur at two sites: either in the ER, after

dimerization with the J chain, or in the extracellular apoplasm, where the secreted antibody is accumulated.

The inherent functions of IgG-constant regions, that is, protein A binding, complement fixation, and the ability to bind to specific cell surface receptors (Fc receptors), may be retained in a dimeric Ig that is capable of binding SC. These additional properties of SlgA-G may enhance the function of the complex in passive immunotherapy, although under some circumstances these biological properties might be undesirable. In principle it should not be difficult to produce a SlgA-G antibody that lacks the C γ 2 domain in these cases.

The development of plants capable of generating functional SlgA may have significant implications for passive immunotherapy. Previously, SlgA has been generated only with difficulty, by in vitro conjugation of SC with dimeric IgA (18) or by the insertion of subcutaneous "backpack" tumors of hybridoma cells secreting monoclonal IgA (19). The plants express SlgA in large amounts, and the production can be scaled up to agricultural proportions. This method offers an economic means of producing large quantities of mAbs that could be applied to mucosal surfaces to prevent infection, as has been demonstrated in passive immunotherapy against streptococci (20). Multivalent antibodies might be more protective than IgG at mucosal surfaces (21), and SC may also have postsecretory functions in stabilizing the polymeric antibody against proteolysis (22). The principle of sexual crossing of transgenic plants to accumulate recombinant subunits can readily be applied to the assembly of a variety of Ig as well as other complex protein molecules.

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